Dominant Role of Hepatitis B Virus and Cofactor Role of Aflatoxin in Hepatocarcinogenesis in Qidong, China

Lihua Ming,[1] Snorri S. Thorgeirsson,[2] Mitchell H. Gail,[3] Peixin Lu,[4] Curtis C. Harris,[2] Nengjin Wang,[4] Yongfu Shao,[1] Zhiyuan Wu,[1] Guoting Liu,[1] Xiaohong Wang,[1] and Zongtang Sun[1]

We assessed the separate and combined effects of hepatitis B virus (HBV), hepatitis C virus (HCV), and aflatoxin in causing hepatocellular carcinoma (HCC) in Qidong, China. A consecutive series of 181 pathologic-diagnosed HCC cases were studied for hepatitis B surface antigen (HBsAg), anti-HBc, HBV X gene sequence, anti-HCV, the 249ser-p53 mutation, and chronic hepatitis pathology. Each of the 181 incident HCC cases had markers for HBV infection and hepatitis pathology; only 6 of 119 cases were coinfected with HCV. The 249ser-p53 mutation was found in 54% (97/181) of HCC cases and in all 7 cases with tissue for analysis from the hepatitis cohort but in none of 42 matched cases from Beijing. The estimated cumulative dose of aflatoxin B1 in these 7 cases ranged from 0.13 to 0.49 mg/kg. Follow-up data through 13.25 years on a cohort of 145 men with chronic HBV hepatitis showed that the relative risk from aflatoxin exposure was 3.5 (1.5-8.1). A similar relative risk was found using 249ser-p53 mutation as a marker for aflatoxin exposure. In conclusion, HBV hepatitis is ubiquitous in Qidong HCC cases, whereas HCV contributes little to its risk. The 249ser-p53 mutation appears to result from coexposure to aflatoxin and HBV infection. Even modest levels of aflatoxin exposure tripled the risk of HCC in HBV-infected men. (Hepatology 2002;36:1214-1220.)

See Editorial on Page 1046

Hepatocellular carcinoma (HCC) is the second most common cancer and kills 300,000 or more people each year in China.[1,2] The close linkage of hepatitis B virus (HBV) to HCC was largely established in epidemiologic studies based on the detection of HBV surface antigen (HBsAg) in sera. In Qidong, China, about 16% of the adult population are seropositive for HBsAg. This marker is not present in every person infected with HBV, however. Early studies had shown that many HBsAg seronegative HCC patients in Qidong had evidence of HBV infection determined by immunohistochemistry[3] or by molecular hybridization in European cases.[4] Fujimoto et al.[5] reported that 23 of 26 HCC cases from Qidong were HBV positive by Southern blot analysis, including 3 HBsAg seronegatives. Paterlini et al.[6] had shown by reverse transcriptase polymerase chain reaction assays that HBV X gene-related transcripts were often found in the diseased and normal hepatic tissues of HBsAg-negative HCC patients. In Japan, many HCC cases attributed to hepatitis C virus (HCV) were found to have markers of HBV infection.[7-9] Zhang et al.[10] reported that PCR analysis of 21 HBsAg seronegative HCC cases in Qidong revealed HBV X gene sequence in the HCC DNA of every case. A recent cohort study in Qidong also showed that HBV worked synergistically with aflatoxin, HCV, and family history to significantly enhance HCC incidence.[11] Using an extensive set of assays, we sought to determine whether HBV infection is virtually ubiquitous in HCC cases in Qidong. Such a finding would have implications for understanding the carcinogenic process

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; AFB1, aflatoxin B1/aflatoxin; AFM1, metabolite M1 of aflatoxin; 249ser-p53, 249 codon arg/ser mutated p53 gene; anti-HBc, antibody to hepatitis B core antigen; RIA, radioimmunoassay; EIA, enzyme immunoassay; anti-HCV, antibody to hepatitis C virus; CAMS, Chinese Academy of Medical Sciences.

From the [1]National Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China; [2]Center for Cancer Research, [3]Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Bethesda, MD; and [4]Qidong Liver Cancer Institute, Qidong, China.

Supported by the National Key Basic Research Project G1998051209 and the National 10th Five-Year Key Scientific Project 2001BA703B06 of Peoples Republic of China.

Address reprint requests to: Zongtang Sun, M.D., National Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Sciences, Panjiayuan, Beijing 100021, China; e-mail: ztsun@public3.bta.net.cn; fax: 8610-6771-3917; or Snorri S. Thorgeirsson, M.D., Ph.D., Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892. E-mail: snorri_thorgeirsson@nih.gov; fax: 301-496-0734.

0270-9139/02/3605-0025$0.00/0
doi:10.1053/jhep.2002.36366

and for strengthening the nationwide HBV vaccination program in China and possibly in other endemic regions.

Exposure to environmental aflatoxin has long been implicated as a risk factor for HCC. Studies of individual levels of aflatoxin in regions with varying HCC risk strengthened the evidence for this association.[12,13] The combination of HBsAg positivity and detectable urinary aflatoxin has been shown to increase the risk of HCC synergistically.[14,15] A missense mutation of codon 249 of the p53 gene that changes arginine into serine (249ser-p53) has been found frequently in HCC cases in areas where HBV and aflatoxin exposure were known risk factors.[16,17] Epidemiologic evidence suggested that this hotspot mutation was strongly associated with and attributable to dietary exposure to aflatoxin.[18-20] However, recent studies in Qidong showed that the high frequency of 249ser-p53 might reflect the action of aflatoxin exposure mainly in persons infected with HBV.[10] Consistent with the notion that HBV sensitizes hepatocytes to the mutagenic effects of aflatoxin was the finding that HCC in HBV-free monkeys fed high doses of aflatoxin did not exhibit 249ser-p53[21] and that this mutation remains prevalent in HCC cases in Qidong despite dietary changes that might reduce aflatoxin exposure in recent decades.[22] The sensitization hypothesis was also supported by the findings that HBV X gene inhibited DNA repair[23] and p53-mediated apoptosis[24] and enhanced the frequency of aflatoxin-induced 249ser-p53 mutations.[25] In this study, we undertook molecular epidemiologic investigations to clarify whether the high prevalence of 249ser-p53 mutations in HCC cases in Qidong was caused by the combined effect of HBV and aflatoxin rather than by aflatoxin alone.

To quantify the effects of HBV, HCV, and aflatoxin on HCC risk in Qidong, we relied on 3 sources of data: (1) molecular studies, including assays for the 249ser-p53 mutation, on 181 consecutive incident HCC cases; (2) measurement of absolute and relative risks of HCC from aflatoxin exposure in a cohort of 145 men with chronic HBV hepatitis; and (3) estimates of attributable risk from aflatoxin among HBV-infected men based on the 249ser-p53 mutation prevalence in incident HCC cases and on urinary measurements of the aflatoxin metabolite AFM1 in Qidong.

Patients and Methods

Study Populations. A consecutive series of 181 incident cases of HCC collected from 1994 to 2000 in the Qidong Liver Cancer Institute in Qidong, Jiangsu Province, China, were diagnosed pathologically and analyzed for HBV and HCV infection status as well as for the presence of 249ser-p53 mutations. In HBsAg-negative HCC cases, assays were conducted to detect antibodies to HBV core antigens (anti-HBc) and/or to detect the HBV X gene sequence. A random sample of 287 sera, collected in a screening survey in Qidong in 1992, was analyzed by radioimmunoassay (RIA) for HBsAg and by Abbott's enzyme immunoassay (EIA) for antibodies to hepatitis C virus (anti-HCV).

We also continued the follow-up to 13.25 years for incident HCC cases in a cohort of 145 HBsAg-positive men with chronic hepatitis and measured aflatoxin exposure as well as HCV, family history, and alcohol consumption status.[11] They had been enrolled and followed at the Qidong Liver Cancer Institute since August 1, 1988. The relative risk of HCC from aflatoxin exposure among these HBV-infected men was calculated directly from the incidence rates in the exposed and unexposed men in this cohort. Pathologic samples were available for measuring the 249ser-p53 mutation from 7 of the 31 patients who developed incident HCC in this cohort, and data were available on each individual's annual average urinary excretion of the AFM1 metabolite of aflatoxin from a pool of at least 8 monthly samples taken in 1987. We compared the prevalence of 249ser-p53 in these 7 HCC patients with that of 42 ethnicity-, age-, and gender-matched (6:1 matching) HBsAg-positive HCC patients in Beijing, who were pathologically diagnosed in the same time period in the Cancer Hospital, Chinese Academy of Medical Sciences (CAMS) in Beijing. Informed consent was obtained from each patient involved, and the study protocol was approved by institutional review boards.

Laboratory Methods. Serum HBsAg in Qidong population was measured using a RIA provided by the Beijing Blood Product Research Institute. Sera in HCC patients in Qidong were analyzed for HBsAg by Abbott AxSym. Anti-HBc and anti-HCV from available frozen (−25°C) sera were also detected in parallel by the Abbott AxSym systems in the Cancer Hospital of CAMS. HBV X gene sequences were detected by nested PCR of the extracted DNA from HCC tissues,[10] and the amplified DNA was sequenced to identify structural features. The 249 codon mutation status of the p53 gene of the HCC DNA was identified from its *Hae*III digestion profile[17] following PCR of the 7th exon of p53 and checked by DNA sequencing in randomly selected cases. The presence of chronic hepatitis was identified by pathologic diagnosis. Exposure to aflatoxin in patients with chronic HBV hepatitis in Qidong was assessed by measuring AFM1 with high-performance liquid chromatography in immunoconcentrated pooled urine collected monthly over a 12-month period in 1987.[11] To estimate daily aflatoxin

B1 intake (in μg/d) from urinary concentrations of AFM1 (in ng/L), we assumed that each subject produced 1 liter of urine daily and multiplied the AFM1 concentration by 50, based on published conversion ratios.[12,26,27] The cumulative dosage of aflatoxin was calculated by multiplying the daily AFB1 intake by the age (in days) at HCC diagnosis, and the cumulative dose per kilogram body weight was based on an assumed weight of 60 kg.

Statistical Methods. To estimate the age-specific incidence rates of HCC in HBsAg-positive men in Qidong, the age-specific HCC incidence rates in the male population were obtained from published sources[28] and adjusted for HBsAg prevalence in the HCC patients' series and in the general population of Qidong. In particular, the estimated incidence of HCC in HBsAg-positive men (Table 3) was calculated as the age-specific HCC incidence in the male population (column 2) times the age-specific prevalence of HBsAg in HCC patients (column 3) divided by the corresponding age-specific prevalence of HBsAg in the general population (column 4). We obtained a 95% confidence interval on the incidence by analyzing its logarithm, which is based on the 3 statistically independent quantities in columns 2, 3, and 4 (Table 3). The variances of these quantities can be estimated by noting that columns 2, 3, and 4 correspond respectively to Poisson, binomial, and binomial variates. Applying the delta method[29] and assuming that the logarithm of the incidence is normally distributed, we obtain a 95% confidence interval for it and, by exponentiation, for the incidence itself.

Confidence intervals and *P* values for odds ratios in 2×2 contingency tables were based on the exact conditional likelihood and on Fisher exact test[30] for independent samples. Tests of equality of proportions in matched samples were based on the method of Mantel and Haenszel,[31] with each matched set regarded as a separate stratum. Estimates of relative risk and attributable risk are based on formulas 2.14 and 2.15, respectively, in *The Analysis of Case-Control Studies*.[32] Numbers separated by a hyphen in parentheses represent 95% confidence intervals. *P* values were based on 2-sided tests

Results

Prevalence of HBV Hepatitis in 181 Incident HCC Cases in Qidong. The incident HCC series consisted of 145 (80%) men and 36 (20%) women. Ages ranged from 27 to 74 years. There were 138 (76%) aged 35 to 54 years and 24 (13%) aged 55 to 64 years.

Of the 181 patients tested for HBsAg by the Abbott AxSym, 171 (94%) were seropositive. Corresponding results for other tests were 128/128 (100%) for anti-HBc and 6/119 (5.0%) for anti-HCV, as shown in Table 1.

Table 1. Prevalence of Markers for HBV Infection, HCV Infection, 249ser-p53 Mutation, and Chronic Hepatitis in HCC Cases From Qidong, China

Viral/Genetic Marker	Prevalence	
	Proportion	Percentage
Serum HBsAg*	171/181	94
Anti-HBc*	128/128	100
HBV marker/s†	181/181	100
Serum anti-HCV*	6/119	5
249arg/ser-p53	97/181	54
Chronic hepatitis‡	181/181	100

*Using Abbott AxSym systems for the relevant marker.
†Positive if any of the markers mentioned in the text is positive.
‡Pathologically diagnosed.

Of the 10 HCC cases that were HBsAg-negative by the Abbott AxSym, all had 1 or more markers indicative of HBV infection. In the 8 cases in which sera were available for further tests, there were signs of infection evidenced by both anti-HBc positivity and presence of HBV X gene sequence. In each of the remaining 2 HCC cases (94-05, 95-33), for whom sera were not available for anti-HBc assays, the HBV X gene sequence was detected by PCR and confirmed by sequencing of the HCC DNA (Fig. 1). HBV X gene sequence from 2 (94-19, 96-18) of the 8 cases with positive anti-HBc but negative HBsAg assays are also shown in Fig. 1. Each detected sequence in Fig. 1 has distinguishing features but bears common variants at codons 130 (nt 1,763-1,765) and 131 (nt 1,766-1,768). These variants had been reported to be quite prevalent in samples from Qidong.[33,10] The anti-HBc assay was positive in every case examined, including 120 HBsAg-positives and 8 HBsAg-negatives.

Thus, all 181 of the pathologic-confirmed HCC cases in this consecutive series in Qidong were associated with markers of HBV. In particular, all 97 cases with 249ser-p53 mutations had evidence of HBV infection, including 5 who were HBsAg-negative by the Abbott AxSym. Relying only on conventional serologic assays for HBsAg would lead to an underestimate of the strength of the association of HBV with HCC in this population. Pathology of the nontumorous liver tissues from resected HCC samples showed features of chronic hepatitis in all 181 HCC cases. There were various amounts of inflammatory cells in the periportal areas, spotty or patchy necrosis, and bridging fibrosis or cirrhosis. Therefore, HBV-related hepatitis was ubiquitous in this consecutive series of HCC cases.

Sera from 119 HCC cases were tested for HCV, and 5% (6/119) had detectable anti-HCV antibodies (Table

```
              1600      1610      1620      1630      1640      1650
D329640*     ACCTCTGCACGTCGCATGGAGACCACCGTGAACGCCCACCAAATATTGCCCAAGGTCTTA
90-8**       .........................................gg.c...............
94-05        .........................................gg.c...............
94-19        ....................a....................gg.c...............
95-33        .........................................gg.c...............
96-18                   ...................g..........gg.c...............

              1660      1670      1680      1690      1700      1710
D329640      CATAAGAGGACTCTTGGACTCTCAGCAATGTCAACGACCGACCTTGAGGCATACTTCAAA
90-8         ............................................................
94-05        ............................................................
94-19        .....................g......................................
95-33        t...........................................................
96-18        .....ac.....................................................

              1720      1730      1740      1750      1760      1770
D329640      GACTGTTTGTTTAAAGACTGGGAGGAGTTGGGGGAGGAGATTAGGTTAAAGGTCTTTGTA
90-8         ..............g..................................t.a........
94-05        ......g.......g..................................t.a........
94-19        ..............g..................................t.a........
95-33        ......g.......g..................................t.a........
96-18        ..............g..................................t.a........
```

Fig. 1. Alignment of HBV X gene sequences from HBsAg sero-negative HCC cases from Qidong, China. *Sequence of HBV X gene segment (nt.1595 to 1774) of HBV from the gene bank.[35] **Sequence of HBV X gene from a HCC sample from Qidong, China.[33]

1). The prevalence of anti-HCV in the general male population of Qidong was estimated to be 1.1% (3/287). In contrast, the prevalence of HBsAg in male HCC cases in Qidong was found to be 95% (137/144), and its prevalence in the general male population was 15.7% (45/287). The odds ratio for HCV exposure in males comparing HCC cases with the general male population was (6/113)/(3/284) = 5.03, which is much smaller than the odds ratio for HBsAg in men with HCC compared with the general population, (137/7)/(45/242) = 105. Moreover, each of the 6 HCC cases infected with HCV was also infected with HBV. Therefore, even though HCV may increase HCC risk among HBsAg-positive individuals (11), the rarity of HCV in this population and its comparatively modest odds ratio imply that only a small proportion of HCC risk is attributable to HCV in Qidong.

Prevalence and Etiology of the 249ser-p53 Mutation in HCC. The 249ser-p53 mutation was identified in 54% (97/181) of the incident HCC cases in Qidong (Table 1). The prevalence of 249ser-p53 in Qidong was similar in men (79/145, 54%) and women (18/36, 50%). As mentioned previously, all 97 of the HCC cases with a 249ser-p53 mutation had laboratory evidence of HBV infection, including the 5 cases who were HBsAg-negative by the Abbott AxSym. It is not surprising, therefore, that the 249ser-p53 prevalence was similar in the HBsAg-positive and HBsAg-negative HCC cases. Five (50%) of the 10 HBsAg-negative HCC cases had the 249ser-p53 mutation.

To understand the high prevalence of the 249ser-p53 mutation in HCC cases in Qidong, we examined the updated follow-up data in a cohort of 145 men with chronic HBV hepatitis and measured aflatoxin exposure[11] and looked for 249ser-p53 mutations in accumulating incident HCC cases with tissue samples. Baseline studies in 1987 and 1988 revealed that urinary AFM1 concentrations in pooled multiple urine samples exceeded the threshold of detection, 3.6 ng/L, in 78 (54%) men. Twenty-four (77%) of the 31 incident HCC cases arising in this cohort through October 31, 2001, had baseline urinary AFM1 concentrations above 3.6 ng/L. Typically, fewer than 10% of HCC cases in Qidong receive surgical treatment. Thus, it is not surprising that tissue samples were available to assay for 249ser-p53 in only 7 of these 31 incident cases. Each of these 7 surgical cases, aged 30 to 50 years, was found to have a 249ser-p53 mutation and to have AFM1 levels well above 3.6 ng/L (Table 2). Two of these 7 patients had recurrent HCC in the 7th and 9th year after the first surgical resection. Samples of HCC from the second operation also carried the 249ser-p53 mutation in contrast to none of 42 ethnicity-, gender-, and age-matched HBsAg-positive HCC patients in Beijing, where exposure to aflatoxin is much less frequent,[12,13] who had the mutation ($P < .0001$). This comparison suggests that HBV alone is not sufficient to

Table 2. Aflatoxin Exposure and p53 Mutation in 7 Incident HCC Cases in the Qidong Hepatitis Cohort

Age (yr)	Average of AFM1 in Urine (ng/L)	AFB1 Exposure Estimated (μg/d)*	Cumulative Dosage (mg/kg)*	Genetic Marker in HCC DNA
43	37.5	1.88	0.49	249ser-p53
45	30.9	1.55	0.42	249ser-p53
49	24.9	1.25	0.37	249ser-p53
50	20.5	1.03	0.31	249ser-p53
30	18.6	0.93	0.17	249ser-p53
46	10.4	0.52	0.15	249ser-p53
49	8.4	0.42	0.13	249ser-p53

NOTE. The estimated aflatoxin B1 intake is obtained by multiplying the urinary AFM1 concentration by 50. This conversion ratio is based on the assumption that each subject produces 1 L of urine per day and on published conversion data.[11,26,27]

*Based on a weight of 60 kg.

induce a 249ser-p53 mutation and that either aflatoxin alone or aflatoxin with HBV infection causes such mutations in HCC in Qidong. In either case, aflatoxin exposure is the common etiologic factor. The ubiquitous existence of HBV infection marker/s, both viral and pathologic, in all of the 97 HCC cases bearing 249ser-p53 supported the notion that the high prevalence of the 249ser-p53 mutation in HCC cases in Qidong is indicative of the carcinogenic effect of aflatoxin exposure in the presence of chronic HBV infection. The lack of even a single HCC case with a 249ser-p53 mutation but without markers of HBV infection indicated that aflatoxin alone in the dosage encountered might not be sufficient to cause HCC in Qidong.

These 7 incident HCC cases had modest urinary concentrations of AFM1, corresponding to daily aflatoxin intakes ranging from 0.42 to 1.88 μg/d (Table 2). From these values and from ages at diagnosis, we calculated the cumulative dose of aflatoxin B1 to be in the range of 0.13 to 0.49 mg/kg body weight (assumed to be 60 kg). This level is approximately 3 orders of magnitude lower than the dose that causes HCC in rhesus monkeys,[34] who were not infected with HBV. Seventy-one percent (17/24) of the incident HCC in the exposed cohort (78 men with AFM1 >3.6 ng/mL) had average urinary AFM1 concentrations below 40 ng/L. These data are consistent with the hypothesis that HBV infection greatly sensitized the infected hepatocytes to the carcinogenic effects of aflatoxin. Among the 78 cohort members with urinary AFM1 levels above 3.6 ng/L, 26 had urinary AFM1 concentrations above 40 ng/L. Among them, 23% (6/26) developed HCC in the follow-up period, compared with 35% (18/52) of the men with urinary AFM1 concentrations below 40 ng/L but above 3.6 ng/L. Thus, among men exposed to aflatoxin, there was no statistically significant difference in HCC incidence between these 2 groups ($P = .54$), and, in fact, those with lower urinary AFM1 levels had a slightly higher HCC incidence. This perturbation of the expected dose-response relationship may reflect sensitization of hepatocytes by HBV infection to the extent that even very low exposures to aflatoxin confer HCC risk.

Estimation of the Relative Risk and Attributable Risk From Aflatoxin in Qidong. After 13.25 years of follow-up, 24 of the 78 men in the chronic HBV hepatitis cohort with baseline AFM1 above 3.6 ng/L developed HCC, compared with 7 of 67 men with AFM1 below this threshold. The incidence rate of HCC in the exposed cohort (AFM1 >3.6 ng/L) was 24 cases/782 person-year = $3{,}069 \times 10^{-5}$ per year, compared with 7 cases/796 person-year = 879×10^{-5} per year in the unexposed cohort. The corresponding relative risk for aflatoxin exposure among HBV-infected men was 3,069/879 = 3.5 (1.5-8.1), and the attributable risk was 57% (16%-72%). These results confirm and make more precise previous estimates of relative and attributable risk from aflatoxin.[11] There was no statistically significant difference in the incidence of fatal cirrhosis between those above the detection threshold for aflatoxin of 3.6 ng/L, 7/78, and those under that threshold, 5/67, ($P = .74$).

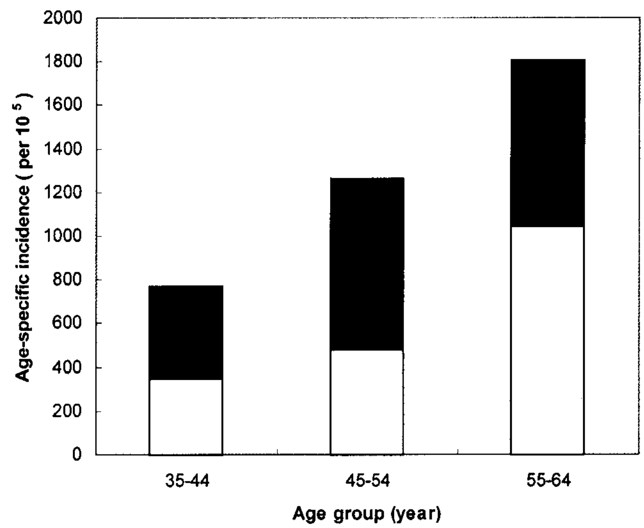

Fig. 2. Age-specific incidence of HCC in HBsAg-positive men of Qidong. **Solid** represents the fraction with a 249ser-p53 mutation.

We used data on the 249ser-p53 mutation in a second approach to quantify the effects of aflatoxin in Qidong. The prevalences of 249ser-p53 in the incident male HCC series were 55% (33/60), 62% (31/50), and 42% (8/19) in the age ranges 35 to 44, 45 to 54, and 55 to 64, respectively. These proportions are indicated in the solid portion in Fig. 2, which depicts HCC incidence rates in HBsAg-positive men in Qidong. These incidence rates, which are shown in column 5 of Table 3, are calculated as age-specific male incidence rates[28] times the HBsAg prevalence in 129 male HCC patients (column 3) divided by the corresponding age-specific HBsAg prevalence in 287 men from general population (column 4). These data suggest that aflatoxin played an etiologic role together with HBV in a large proportion of the HCC cases in Qidong. To calculate the attributable risk from aflatoxin in HBV-infected men, we assumed that cases with the mutation had been exposed to aflatoxin levels such that urinary AFM1 concentrations exceeded 3.6 ng/L, whereas cases without the mutation had lower or no exposure. We also used an estimate of the prevalence of exposure to aflatoxin (AFM1 >3.6 ng/L) derived from a small survey[13] of 40 men in the general population of Qidong in 1983, namely 27.5% (11/40). We note that this estimate is quite consistent with a prevalence of aflatoxin exposure of 55.8% in male HCC cases (based on

Table 3. Age-Specific Incidence Rates of HCC in HBsAg Seropositive Men in Qidong

Age Range (yr)	HCC Incidence per 10^5 Inhabitants of Qidong	HBsAg Prevalence in HCC Patients	HBsAg Prevalence in Population	HCC per 10^5 in HBsAg-Positive Men (range)
35-44	168	98% (59/60)	21.4% (24/112)	769 (537-1,110)
45-54	199	96% (48/50)	15.1% (14/93)	1,265 (777-2,082)
55-64	172	90% (17/19)	8.57% (6/70)	1,806 (820-3,951)

245ser-p53 mutation data) and with a relative risk of 3.5 (as in the hepatitis cohort), which together imply a prevalence of exposure in the control population of 26.5%. The resulting estimates of relative risk were 3.2 (1.3-8.4), 4.3 (1.6-11.8), and 1.9 (0.52-6.9) in the age ranges 35 to 44, 45 to 54, and 55 to 64 years, respectively. The corresponding attributable risks were 38% (15%-56%), 48% (22%-65%), and 20% (0%-48%), respectively. These attributable risks are smaller (though not statistically significantly so) than for the hepatitis cohort mainly because the proportions exposed to AFM1 >3.6 ng/L are smaller in the general population (27.5% vs. 54%).

Discussion

Each of the 181 incident HCC cases in this consecutive series from Qidong arose in association with HBV hepatitis, as determined by pathologic examination and by testing for HBsAg, anti-HBc, and HBV X gene sequences. Previous studies based only on HBsAg could underestimate the essential role of HBV infection in hepatocarcinogenesis in Qidong. In contrast, anti-HCV antibodies were detected in only 5% of the HCC cases tested, each of whom was also infected with HBV, and the odds ratio associated with HCV was 20-fold lower than that for HBV. Thus, HBV is the predominant predisposing factor for HCC in this population.

HBV poses a high level of risk for HCC by itself (odds ratio 105), and joint risks from exposure to other factors, such as a positive family history, HCV infection, and aflatoxin, were found to be even higher in a previous study of the cohort of men in Qidong with hepatitis.[11] We were particularly interested in studying aflatoxin as a cofactor of increased HCC risk in HBV-infected subjects because of the potential to control aflatoxin exposure in the sizeable HBV-infected population in Qidong and other areas of high prevalence. Ninety-seven (54%) of the 181 incident HCC cases in Qidong carried the 249ser-p53 mutation, a marker for joint exposure to HBV and aflatoxin, whereas this marker was not found in any of the 42 matched HCC cases from Beijing, where persistent aflatoxin exposure is infrequent.[12,13]

We analyzed recent follow-up information from a cohort of 145 men with chronic HBV hepatitis. We defined a subject as exposed to aflatoxin if the urinary AFM1 concentration exceeded 3.6 ng/L. Aflatoxin exposure was associated with a relative risk of 3.5 (1.5-8.1) and an attributable risk of 57% (16%-72%) in this cohort.

It is tempting to interpret the 249ser-p53 marker data in Fig. 2 as indicating that about half the HCC in the general population of Qidong is attributable to aflatoxin acting on HBV-infected persons. More formal analyses found in the Results section indicate that aflatoxin and HBV coexposure may account for perhaps 40% of HCC incidence in the general population of Qidong in the age range 35 to 54 years, in which most cases occur.

Efforts to control aflatoxin exposure must take into account the evidence suggesting that HBV sensitizes hepatocytes to the carcinogenic effects of aflatoxin. Each of the 7 incident HCC cases in the hepatitis cohort who were assayed for 249ser-p53 was found to carry the mutation, and their baseline aflatoxin B1 intakes ranged from 0.42 to 1.88 μg/d. The cumulative intakes up to the age of HCC diagnosis were calculated to range from 0.13 mg/kg to 0.49 mg/kg, assuming a weight of 60 kg. These levels are about 1,633- to 415-fold lower than required to induce HCC in HBV-free rhesus monkeys, for whom a carcinogenic dose was reported to be 245 mg/kg via oral administration.[34] Similar levels of cumulative aflatoxin intake were found for the majority of incident HCC cases in the members of the hepatitis cohort with baseline AFM1 concentrations above 3.6 ng/L, substantiating that HBV infection can sensitize human hepatocytes to the effects of aflatoxin at low dosage. This dosage is below the 5 ppb dietary contamination level used as a standard by the World Health Organization and Chinese. For patients with chronic HBV hepatitis, a small percentage of the population, it seems prudent to recommend foods low in aflatoxin and to monitor urinary AFM1 levels.

References

1. Li LD, Lu FZ, Zhang SW, Mu R, Sun ST, Huangfu XM. Distribution analysis of cancer mortality in China during 1990-1992. Chin J Oncol 1996;18:403-407.
2. Sun ZT, Ming LH, Zhu X, Lu JH. Prevention and control of hepatitis B in China. J Med Virol 2002;67:447-450.
3. Xia QJ, Li TS, Li YS, Sun ZT, Wang NJ, Shao LQ, Zhang SP. Liver cell cancer in young adolescent and its relation to hepatitis B virus. Chin J Oncol 1984;6:413-416.
4. Brechot C, Dogos F, Lugassy C, Thiers V, Zafrani S, Franco D, Bismuth H, et al. Hepatitis B virus DNA in patients with chronic liver disease and negative tests for hepatitis B surface antigen. N Engl J Med 1985;312:270-276.
5. Fujimoto Y, Hampton LL, Wirth PJ, Wang NJ, Xie JP, Thorgeirsson SS. Alterations of tumor suppressor genes and allelic losses in human hepatocellular carcinomas of China. Cancer Res 1994;54:281-285.
6. Paterlini P, Poussin K, Kew M, Franco D, Brechot C. Selective accumulation of the x transcript of hepatitis B virus in patients negative for hepa-

titis B surface antigen with hepatocellular carcinoma. HEPATOLOGY 1995; 21:313-321.
7. Koike K, Nakamura Y, Kobayashi M, Takada S, Urashima T, Saigo K, Kobayashi S, et al. Hepatitis B virus DNA integration frequently observed in the hepatocellular carcinoma DNA of hepatitis C virus-infected patients. Int J Oncol 1996;8:781-784.
8. Urashima T, Saigo K, Kobayashi S, Imaseki H, Matsubara H, Koide Y, Asano T, et al. Identification of hepatitis B virus integration in hepatitis c virus-infected hepatocellular carcinoma tissues. J Hepatol 1997;26:771-778.
9. Sugawara Y, Makuuchi M, Takada K. Detection of hepatitis B virus DNA in tissues of hepatocellular carcinomas related to hepatitis C virus, which are negative for hepatitis B virus surface antigen. Scand J Gastroenterol 1999;9:934-938.
10. Zhang F, Zhu YY, Sun ZT. Universal presence of HBV X gene and its close association with hotspot mutation of p53 gene in hepatocellular carcinoma of prevalent area in China. Chin J Oncol 1998;20:18-21.
11. Sun ZT, Lu PX, Gail MH, Pee D, Zhang QN, Ming LH, Wang NJ, et al. Increased risk of hepatocellular carcinoma in male HBsAg carriers with chronic hepatitis who have detectable urinary aflatoxin metabolite M1. HEPATOLOGY 1999;30:379-383.
12. Sun T, Chu Y. Carcinogenesis and prevention strategy of liver cancer in area of prevalence. J Cell Physiol Suppl 1984;3:39-44.
13. Sun T, Wu S, Wu Y, Chu Y. Measurement of individual aflatoxin exposure among people having different risk to primary hepatocellular carcinoma. In: Hayashi Y, Nagao M, Sugimura T, Takayama S, Tomatis L, Wattenberg LW, et al., eds. Diet, Nutrition, and Cancer. Proc 16th Int Symp of Princess Takamatsu Cancer Res Fund. Tokyo: Japanese Science Society Press, 1986;225-235.
14. Ross RK, Yuan J-M, Yu MC, Wogan GN, Qian G-S, Tu J-T, Groopman JD, et al. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. Lancet 1992;339:943-946.
15. Qian G-S, Ross RK, Yu MC, Yuan J-M, Gao Y-T, Henderson BE, Wogan GN, et al. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. Cancer Epidemiol Biomark Prevent 1994;3:3-10.
16. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 1991;350:427-428.
17. Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature 1991;350: 429-431.
18. Ozturk M. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. Lancet 1991;338:1356-1359.
19. Kirk GD, Carmus-Randon AM, Mendy M, Goedert JJ, Merle P, Trepo C, Brechot C, et al. Ser-249 p53 mutation in plasma DNA of patients with hepatocellular carcinoma from the Gambia. JNCI 2000;92:148-153.
20. Shimizu Y, Zhu JJ, Han F, Ishikawa T, Oda H. Different frequencies of p53 codon-249 hotspot mutations in hepatocellular carcinomas in Jiangsu Province of China. Int J Cancer 1999;82:187-190.
21. Fujimoto Y, Hampton L, Luo L, Wirth PJ, Thorgeirsson SS. Low frequency of p53 gene in tumors induced by aflatoxin B1 in nonhuman primates. Cancer Res 1992;52:1044-1046.
22. Ming LH, Yuan BZ, Thorgeirsson SS, Zhu YR, Wu ZY, Sun ZT. Characteristics of high frequency 249 codon mutation of p53 gene in hepatocellular carcinoma of prevalent area of China. Chin J Oncol 1999;21:122-124.
23. Jia L, Wang XW, Harris CC. Hepatitis B virus X protein inhibits nucleotide excision repair. Int J Cancer 1999;80:875-879.
24. Elmore LW, Hancock AR, Chang SF, Wang XW, Chang S, Callahan CP, Geller DA, et al. Hepatitis B virus X protein and p53 tumor suppressor interactions in the modulation of apoptosis. Proc Natl Acad Sci U S A 1997;95:14707-14712.
25. Sohn S, Jaitovitch-Groisman I, Benlimame N, Galipeau J, Batist G, Alaoui-Jamali MA. Retroviral expression of the hepatitis B virus x gene promotes liver cell susceptibility to carcinogen-induced site specific mutagenesis. Mutat Res DNA Repair 2000;460:17-28.
26. Zhu JQ, Zhang LS, Hu X, Xiao Y, Chen JS, Xu YC, Fremy J, et al. Correlation of dietary aflatoxin B1 levels with excretion of aflatoxin M1 in human urine. Cancer Res 1987;47:1848-1852.
27. Chen Z, Root M, Pan W, Chen J, Campbell TC. Use of an improved method for analysis of urinary aflatoxin M1 in a survey of mainland China and Taiwan. Cancer Epidemiol Biomark Prevent 1977;6:523-529.
28. Chen JG, Li WG: Cancer incidence in Qidong, China 1988-1992. In: Parkin DM, Whelan SL, Ferlay J, Raymond L, Young J, eds. Cancer Incidence in Five Continents. Volume VII. Lyon: IARC Sci Publ No. 143, 1997;322-325.
29. Rao CR. Linear Statistical Inference and Its Applications. New York: Wiley, 1966:319-322.
30. Mehta C, Patel N. StatXact3 for Windows Users Manual, Chapter 13. Cambridge, MA: Cytel Software Corp., 1995.
31. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. J Natl Cancer Inst 1959;22:719-748.
32. Breslow NE, Day NE. Statistical Methods in Cancer Research. Volume 1. The Analysis of Case-Control Studies. Lyon: IARC, 1980.
33. Hsia CC, Nakashima Y, Tabor E. Deletion mutants of the hepatitis B virus X gene in human hepatocellular carcinoma. Biochem Biophys Res Commun 1997;241:726-729.
34. Adamson RH. Induction of hepatocellular carcinoma in non-human primates by chemical carcinogens. Cancer Detect Prevent 1989;14:215-219.
35. Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature 1979;281:646-650.